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14. ABSTRACT Mucin1 (MUC1), a glycoprotein is aberrantly overexpressed in TNBC and facilitates growth and metastasis of triple negative breast cancer (TNBC) cells. This occurrence can be partially attributed to MUC1 interaction with hypoxia-inducible factor alpha (HIF1 α), a key regulator of glycolysis. We previously observed that ectopic overexpression of MUC1 increased glucose uptake, lactate secretion and enhanced the expression of glycolytic enzymes. Therefore we hypothesized that MUC1 stabilizes HIF1 α to facilitate metabolic reprogramming. In the present study we examined the effect of MUC1 expression on cancer cell metabolism of TNBC cell lines. MUC1 was ectopically overexpressed in the MDA-MB231 cell line and stably knocked down in the MDA-MB468 and BT-20 cell lines. Results indicate that MUC1 expression altered the expression of several metabolic genes. Furthermore, untargeted global metabolomic profiling identified metabolite alterations in which MUC1 expression modulates cancer cell metabolism to facilitate growth properties of TNBC cells. Thus our results support the notion that MUC1 serves as a metabolic regulator in TNBC, facilitating metabolic reprogramming that influences growth of TNBC.					
15. SUBJECT TERMS Cancer metabolism, glycolysis, mucin1, pentose phosphate pathway, triple negative breast cancer					
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1. INTRODUCTION

Breast cancer, the second leading cause of cancer deaths in women, is the most common cancer among North American women, accounting for nearly 1 in 3 cancer cases diagnosed in the U.S. women [1, 2]. Triple negative breast cancer (TNBC) subtype accounts for approximately 15-25% of all breast cancer cases and has an increased incidence of metastasis, high recurrence within 1-3 years and a high mortality rate [3]. Therefore, identifying factors that facilitate tumor growth and metastases have the potential to serve as novel molecular targets for breast cancer therapy. MUC1, a glycoprotein associated with chemoresistance, is aberrantly overexpressed in over 90% of early TNBC lesions [4-6]. Much of the oncogenic potential role of MUC1 can be attributed to the participation of the small, cytoplasmic tail of MUC1 (MUC1 CT) in signal transduction and transcriptional events, facilitating growth and metastasis [6-9]. Oncogenic potential can also be attributed to MUC1 ability to interact and stabilize hypoxia-inducible factor alpha (HIF1 α), a key regulator of glycolysis [10]. As metastasis is the leading cause of cancer related deaths, this process relies on cooperation between the tumor cells and their surrounding stroma, establishing a reactive tumor microenvironment. Stromal cells can serve as a sink for the end-products of aerobic glycolysis (i.e., lactate) and provide a source of metabolites (i.e., pyruvate) to support cancer growth, invasion and metastasis [9]. Hence our overall research focus is to investigate how signaling through MUC1 facilitates hypoxia-dependent and independent metabolic cross-talk between epithelial and stromal components in TNBC; thus facilitating tumor growth and metastasis. Additionally, we will examine if co-targeting MUC1 and HIF1 α will block epithelial-stroma metabolic cross-talk, diminish chemoresistance and reduce tumor growth and metastasis in TNBC. Findings from the proposed study may identify MUC1 as a novel therapeutic target for breast cancer, particularly for the TNBC subtype.

2. KEYWORDS: cancer metabolism, glycolysis, mucin1, pentose phosphate pathway, triple negative breast cancer

3. OVERALL PROJECT SUMMARY

Specific Aim 1

Research Objective: To elucidate the mechanism by which MUC1 modulates tumor-stromal metabolic cross-talk, promoting tumor growth and metastasis in distinct triple-negative breast cancer microenvironment.

Methodology

Cell Culture

MDA-MB231 (Neo and MUC1), MDA-MB468 (NEO and shMUC1), and BT20 (NEO and shMUC1) cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μ g/ml streptomycin. Cells were maintained in a humidified atmosphere at 37 °C with 5% CO₂ under atmospheric oxygen condition (20%).

Hypoxia To generate hypoxic conditions, the oxygen concentration was set to 1% and the cells were maintained under hypoxia for 24 hours.

Generation of Conditioned Media (CM)

To generate conditioned media, cells were cultured in DMEM supplemented with 5% dialyzed FBS (dFBS). After 24 h, the CM was harvested and centrifuged at 3000 g for 5 min to remove cell debris then passed through a 0.22 μ m filter. The CM was stored at -80°C until use and was used alone or diluted 1:1 with DMEM supplemented with 5% dFBS.

MTT Cell Viability Assay

The viability of cultured cells was determined by assaying the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide [MTT (Sigma-Aldrich, St. Louis, MO)] to formazan. Cells were seeded at 3×10^4 cell/well in 96-well plates in a final volume of 100 μ l, incubated overnight and then incubated for 72 hours under indicated conditions. Twenty microliters of MTT reagent (diluted in culture media) was added to each well and cells were incubated for 4 hr at 37°C . The MTT formazan precipitate was then dissolved in 100 μ l of DMSO, and the absorbance was measured at 570 nm using a Cytation 3, plate reader (Biotek, USA).

Quantitative Real-Time Polymerase Chain Reaction

Quantitative real-time polymerase chain reaction (qPCR) was performed in 384 Well Optical Reaction Plates (Applied Biosystems) using SYBRGreen PCR Master Mix (Roche). Reactions were carried out on an ABI 7500 thermocycler (Applied Biosystems). All samples were amplified in duplicate and quantification of the expression level of each gene was calculated using the delta delta CT method, normalized to β -actin. Data presented as fold change relative to the control cells (Neo).

Glucose Uptake Assay

5×10^4 cells were seeded per well in a 24-well plate and allowed to adhere. Cells were then serum starved for 24 h and stimulated with EGF at the indicated concentration and time. Next cells were starved for glucose for 2 h and then incubated for 20 min with $1 \mu\text{Ci}$ [^3H]-2-deoxyglucose and lysed with 1% SDS. The lysates were counted for [^3H] using a scintillation counter. Cells treated with labeled and excess unlabeled glucose were used as controls to set a baseline for nonspecific tritium uptake. The results were normalized to the cell counts for normoxia and hypoxia groups. Data are presented as the mean value of quadruplicate values of glucose uptake normalized with control cells.

Glutamine Uptake Assay

5×10^4 cells were seeded per well in a 24-well plate and allowed to adhere overnight. For hypoxic conditions cells were maintained under hypoxia for 12 hours following overnight incubation. Cells were incubated for 2 min with $3 \mu\text{Ci}$ [^3H]-glutamine then lysed with 1% SDS. The lysates were counted for [^3H] using a scintillation counter. Cells treated with labeled and excess unlabeled glutamine were used as controls to set a baseline for nonspecific tritium uptake. The results were normalized to the cell counts for normoxia and hypoxia groups. Data are presented as the mean value of quadruplicate values of glutamine uptake normalized with control cells.

Liquid chromatography and tandem mass spectrometry for polar metabolites

Cells cultured for 24 h were rinsed with PBS then frozen on dry ice for metabolite extraction. Extracts were collected by the addition of 80% methanol on dry ice followed by plates were maintained at -80°C for 10 min and lysed using cell scrapers. Lysates were collected in polypropyl

tubes and centrifuged at 3400 rpm at 4°C for 10 min to remove the precipitates. Pellets were suspended in 200 µl of LC-MS grade water and centrifuged to collect the water soluble supernatants. The combined supernatants were concentrated for 1h using speed vacuum concentrator followed by lyophilization for 2h using Freezone (-105°C) lyophilizer (Labconco). Lyophilized concentrates were suspended in equal volumes of LC-MS grade water and 10 µl were utilized for LC-MS/MS using multiple reaction monitoring (MRM) method described previously [21]. Data acquisition was carried out using Analyst™1.6 software (AB SCIEX) and peaks were integrated with Multiquant™ (AB SCIEX). Peak areas were normalized with the respective protein concentrations and the resultant peak areas were subjected to Metaboanalyst.2 for relative quantification analyses.

Statistical Analysis: Nonparametric Tukey tests were used to compare differences between cell lines. A p-value of 0.05 or less will be deemed significant.

Results

MUC1 expression induces metabolic alterations in TNBC cells

Our previous data showed that MUC1-expression alters metabolic gene expression in MDA-MB231 TNBC cells when MUC1 was overexpressed. To further determine the role that MUC1 plays in metabolic alterations stable knockdown of MUC1 was established in TNBC cells MDA-MB468 and BT20 that overexpress the glycoprotein (Figure 1). Next to determine if MUC1 regulates expression of glycolytic and TCA cycle genes quantitative real-time polymerase chain reaction analysis was utilized. Results indicated that several genes in each pathway were altered comparing MDA-MB468 and BT20 (shMUC1) with control MDA-MB468 and BT20 (NEO) cells (Figure 2). Furthermore to determine if MUC1 expression results in differential metabolism, we performed metabolomics using LC-MS/MS platform and identified metabolic differences between control cells (NEO) and experimental cells (MUC1 or shMUC1) cells. PLS-DA analysis of the polar metabolite component indicated overall metabolic distinction between control and experimental cells (Figure 3). Pathway enrichment analyses showed that control and experimental cells had differential alterations in metabolic pathways (Figure 4). Galactose and tryptophan metabolism were most significantly altered in MDA-MB231 closely followed by glycolysis pathway. Protein biosynthesis and ammonia recycling were most significantly altered in MDA-MB468 closely followed by citric acid cycle pathway. Mitochondrial electron transport chain and citric acid cycle pathways were most significantly altered in BT20. Furthermore, relative comparison of the fold change in individual metabolite levels within glycolysis, TCA cycle and pentose phosphate pathway (PPP) showed a 2 fold differences in most of the metabolites between control and experimental groups (Figure 5).

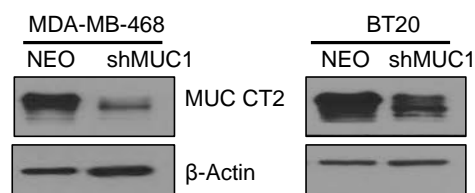


Figure 1. MUC1 knockdown in TNBC cells. Immunoblot analysis of MUC1 CT2 expression following stable knockdown. β actin serves as the loading control.

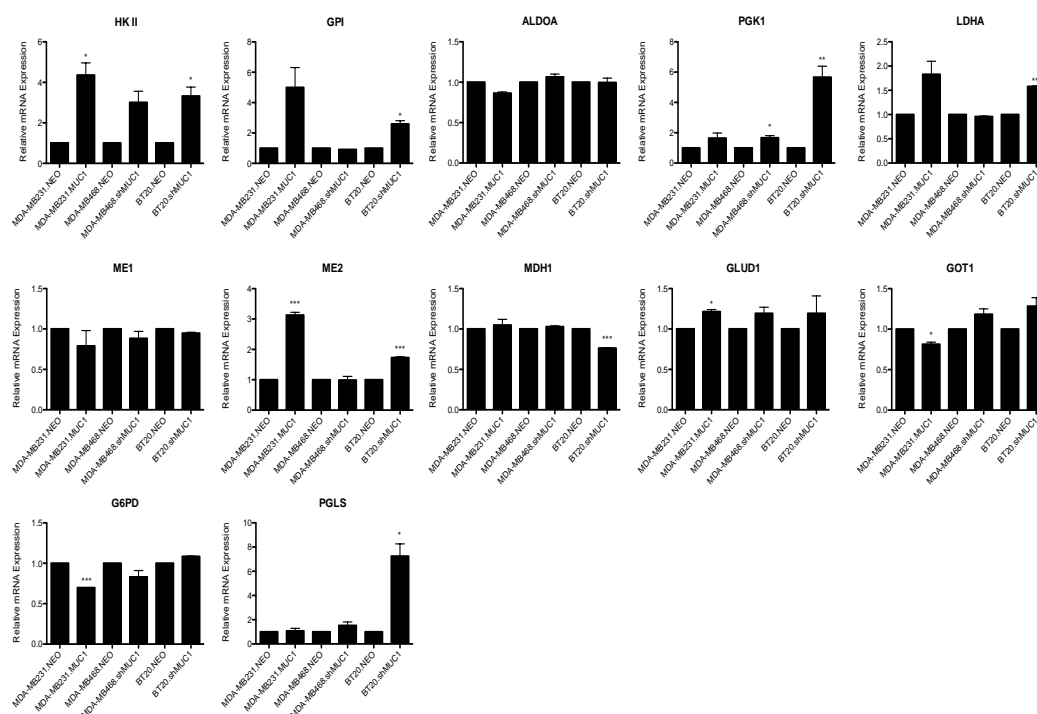


Figure 2. MUC1 regulates metabolic gene expression in TNBC cells. Gene expression analysis of indicated gene comparing experimental cells (MUC1 or shMUC1) with control (NEO) cells.

Differential Metabolomics

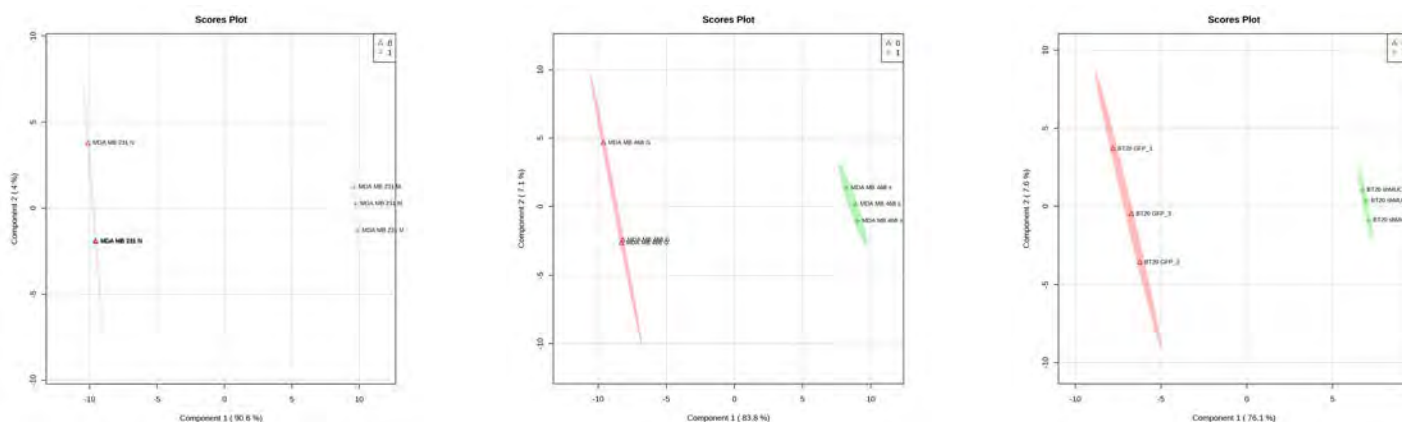
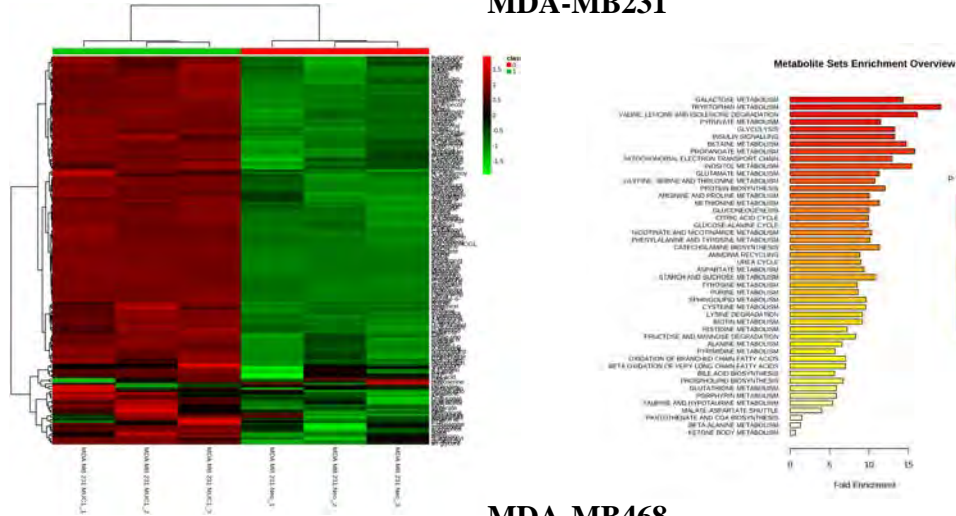
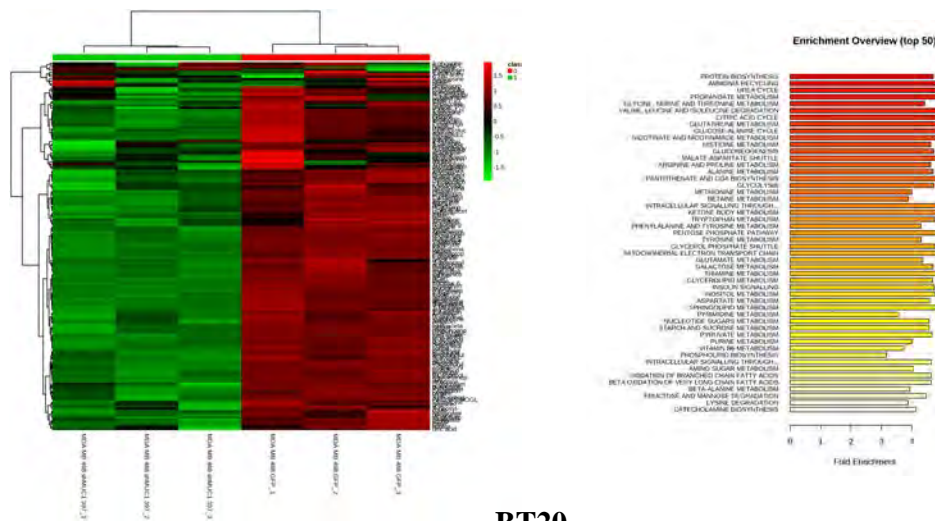


Figure 3. Partial least square Discriminant analysis (PLS-DA) model. Analysis was performed using MetaboAnalyst 2.0 on differentially expressed metabolites of the control (red) and experimental (green) groups. Circles represent 95% confidence interval for similarities in metabolite profiles.

MDA-MB231



MDA-MB468



BT20

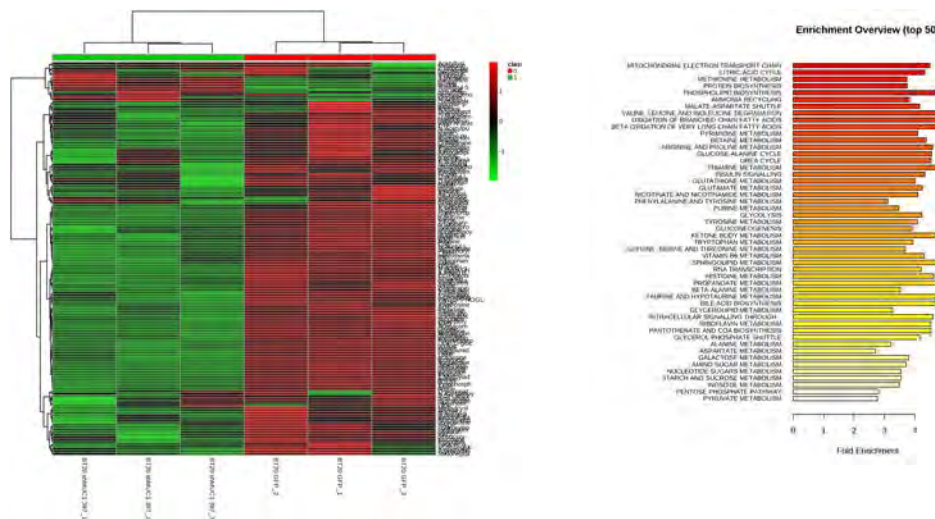
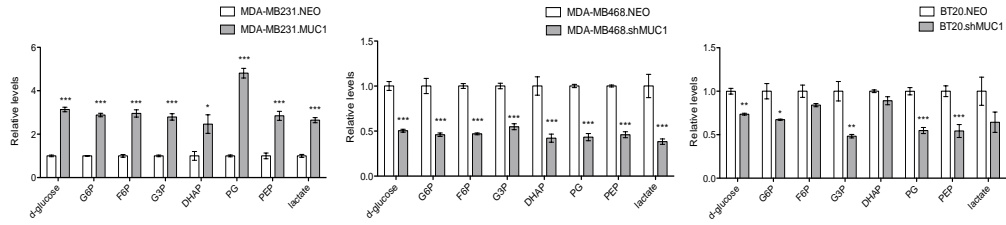
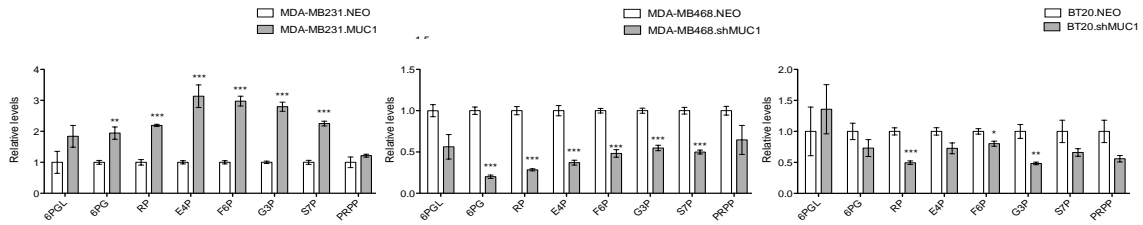


Figure 4. MUC1 regulates metabolite Levels in TNBC cells. Heat map (left) generated from the normalized-mean peak intensities for each metabolite identified from triplicate sets, from 0%(red) to 100% (green). Summary plot (left) for quantitative enrichment analysis (QEA) of significantly altered metabolites in control group as compared to experimental group. Analysis was performed using MetaboAnalyst 2.0.

Glycolysis



Pentose Phosphate Pathway



TCA Cycle

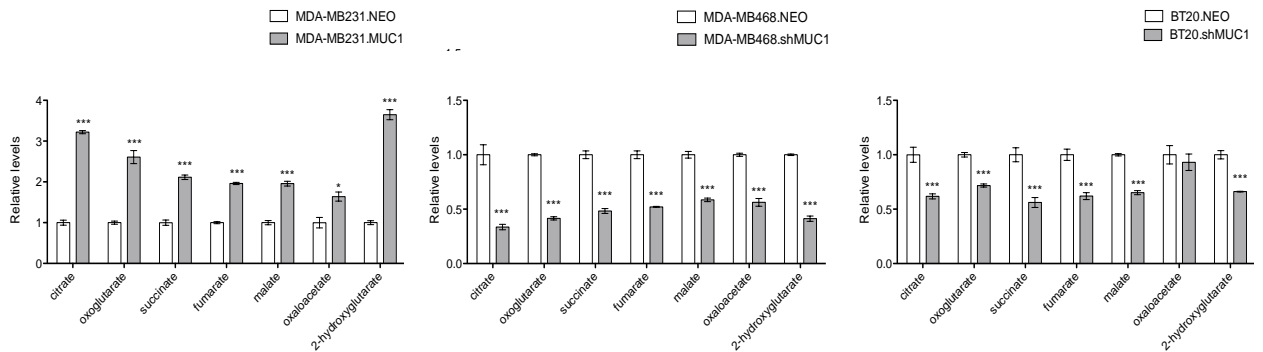


Figure 5. MUC1 regulates metabolite expression in TNBC cells. Relative quantification of indicated metabolite within glycolysis, pentose phosphate pathway and TCA cycle, comparing control with experimental group.

Human breast stromal cell condition media alters cell growth in TNBC cells

Breast cancer progression is promoted by stromal cells that populate the tumors microenvironment which including human mammary fibroblast (hMF). Therefore the effect of conditioned media (CM) from hMF stromal cells on the cell growth of breast cancer cells was investigated (Figure 6). Cell viability assays revealed that CM had increased cell growth compared to control conditions (5%dFBS) only in MDA-MB468 cells. Additionally dilution of the CM media reduced this effect. It has previously been shown that tumors exhibit increased dependence on glycolysis, resulting in abundant export of lactic acid. Lactic acid is mainly transported by two H⁺/lactate symporters, MCT1/MCT4, that require the cell surface glycoprotein, CD147/Basigin for their function. Therefore, the effect of CM from hMF stromal cells with CD147 knockdown on the cell growth of breast cancer cells was also investigated. As shown in Figure 6, CD147 knockdown has no effect on CM induced cell growth of TNBC cells. These findings demonstrate that proteins and metabolites secreted by stromal cells impart a growth advantage, providing further support of cross-talk between epithelial and stromal cells in TNBC cells.

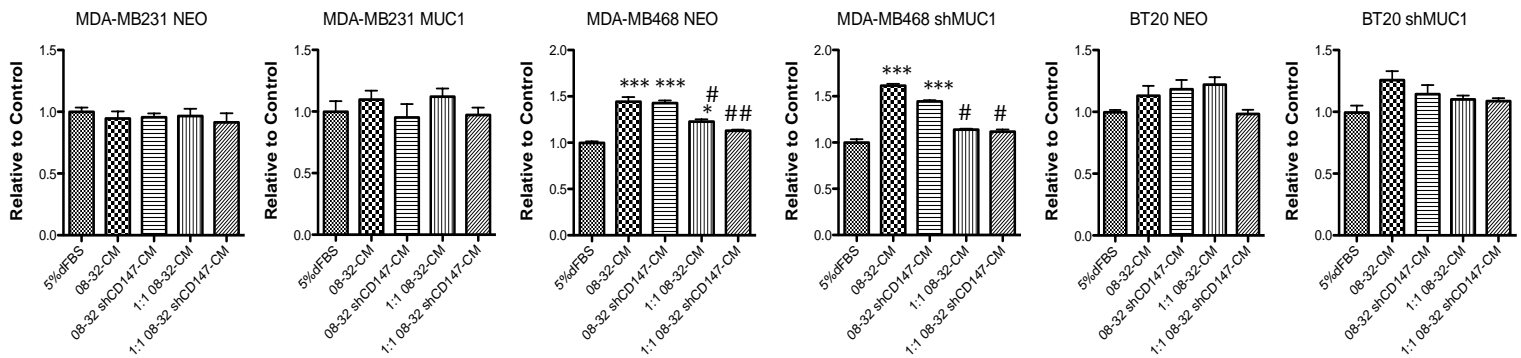


Figure 6. Human mammary fibroblast cell condition media alters TNBC cell growth. Growth of TNBC cells (72h) incubated with hMF stromal cell condition media (24h). * $p < 0.05$ vs 5%dFBS # $p < 0.05$ vs indicated undiluted CM

MUC1 regulates glucose and glutamine uptake in TNBC cells

Glucose and glutamine have been shown to play a role in promoting cancer growth, participating in energy formation and redox homeostasis [12]. It was previously shown that MUC1 overexpression increased glucose and glutamine uptake. Therefore glucose and glutamine uptake assay was utilized to determine the effect of MUC1 knockdown on [³H]-2DG and [³H]-glutamine uptake. Results showed that MUC1 knockdown significantly reduced glucose and glutamine uptake in MDA-MB468 cells however no significant reduction was seen in BT20 cells (Figure 7). These results indicate that MUC1 facilitates the uptake of both glucose and glutamine as a carbon sources.

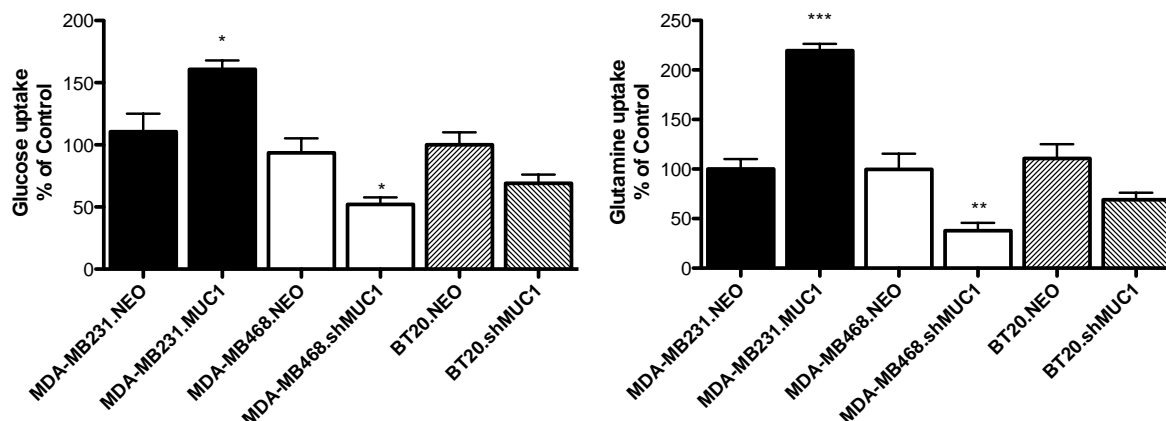


Figure 7. MUC1 regulates glucose and glutamate in TNBC cells. (Left) 3 μ Ci [3 H]-2DG uptake and (Right) 3 μ Ci [3 H]-glutamine uptake. * p <0.05, ** p <0.01 and *** p <0.001

Discussion

In summary, our results support the notion that MUC1 serves as a metabolic regulator in TNBC. Glucose and glutamine serve as the main carbon sources in proliferating cells, and uptake of both nutrients is directed by growth factor signaling[13]. Examining expression of genes regulating metabolic processing revealed MUC1 facilitated alteration in a number of the genes regulating glycolysis and TCA cycle (Figure 2). Next, untargeted global metabolomic profiling identified metabolite alterations in which MUC1 expression modulates cancer cell metabolism to facilitate growth properties of TNBC cells (Figures 4-5). Using pathway enrichment analyses the top altered pathways were identified that include: galactose metabolism, tryptophan metabolism, glycolysis, citric acid cycle and mitochondrial electron transport chain pathway. These pathways have all been implicated in supporting cancer growth. Additionally, the observed increase in cell growth in the presence of hMF CM highlights the importance of the epithelial-stroma metabolic cross-talk in which stromal cells can serve as a sink for the end-products of aerobic glycolysis (i.e, lactate) and provide a source of metabolites (i.e., pyruvate) to support cancer growth. This is further supported by the observed decrease in TNBC cell growth comparing hMF CM with CM from hMF exhibiting CD147 knockdown. Lastly, observed alterations in glucose and glutamine uptake (Figure 7) suggest MUC1 facilitates the utilization of both glucose and glutamine as carbon sources to maximize ATP production in TNBC.

Actual or anticipated problems

For our remaining experiments we will utilize methodologies that are well established in our laboratory and/or the laboratories of collaborators, therefore we do not anticipate future problems.

4. KEY RESEARCH ACCOMPLISHMENTS

- Determined alterations of key genes regulating metabolic processes facilitated by MUC1
- Determined MUC1 regulates metabolite expression in TNBC cells

- Determined glucose and glutamine uptake is altered by MUC1 expression
- Determined human breast stromal cell condition media alters cell growth in TNBC cells
- Determined CD174 regulates tumor-stromal crosstalk to promote cell growth

5. CONCLUSION

In conclusion, the data presented here establishes a role of MUC1 in metabolic reprogramming in TNBC. Presented data suggest that MUC1 facilitates metabolic reprogramming of TNBC to promote cell growth, which can in part be due to the interaction of MUC1 with HIF1 α . PLS-DA indicates clustering into two separate groups that can be metabolically differentiated. In addition, pathway analysis indicates that MUC1 is involved multiple metabolic pathways, altering the expression of several metabolites. Our findings also highlight the potential of targeting MUC1 for metastatic breast cancer therapy. Within the next year we will continue co-culture experiments with human mammary fibroblast (hMF) already obtained. Additionally, we will establish stably mCherry expressing hMF and luciferase expressing TNBC cells in order to initiate *in vivo* studies.

6. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:

- List all manuscripts submitted for publication during the period covered by this report resulting from this project. Include those in the categories of lay press, peer-reviewed scientific journals, invited articles, and abstracts. Each entry shall include the author(s), article title, journal name, book title, editors(s), publisher, volume number, page number(s), date, DOI, PMID, and/or ISBN.
 - (1) Lay Press: Nothing to report
 - (2) Peer-Reviewed Scientific Journals: Nothing to report
 - (3) Invited Articles: Nothing to report
 - (4) Abstracts: Goode, G. Chaika, N and Singh, PK “MUC1 serves as metabolic regulator in triple negative breast cancer”. doi: 10.1158/1538-7445.AM2015-1838 *Cancer Res* August 1, 2015 75; 1838
- List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.

Poster presentation, “MUC1 serves as metabolic regulator in triple negative breast cancer”. Presented at the American Association for Cancer Research (AACR) Annual meeting, Philadelphia, PA April 18-22, 2015

7. INVENTIONS, PATENTS AND LICENSES: Nothing to report

8. REPORTABLE OUTCOMES: Nothing to report

9. OTHER ACHIEVEMENTS: Nothing to report

For each section, 4 through 9, if there is no reportable outcome, state “Nothing to report.”

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10. APPENDICES: Nothing to report